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In re Application of:	)	Conf. No.: 5965
	)	
Emma KVITNITSKY et al	)	Art Unit:
	)	
Appln. No.: 10/553,757	)	Examiner: Nizal S. Chandrakumar
	)	
Filed: January 3, 2007	)	Washington, D.C.
	)	
For: STABILIZED DERIVATIVES	)	
OF ASCORBIC ACID	)	

DECLARATION OF DR. BELAKHOV

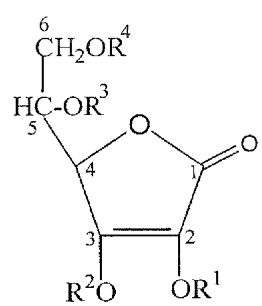
I, the undersigned Dr. Valery Belakhov, hereby solemnly declare as follows:

I am one of the inventors of the invention of the above-identified application, and am very familiar with its contents. Attached is an abbreviated version of my *Curriculum Vitae* of August 2009, which reveals my education, my work experience and my recent publications.

Introduction

The claims of the present invention define a compound of the general formula I:

I



wherein R^1 is a C_8-C_{18} acyl group, an amino acid group, or a C_1-C_{17} alkyl group; R^2 is ammonium, a monovalent metal cation of Na^+ or K^+, a divalent alkaline earth metal cation of Mg^{++}, Ca^{++} or Ba^{++}, or a trivalent metal cation of Al^{+++} or Fe^{+++}; and each of R^3 or R^4, independently, is hydrogen, a C_2-C_{22} acyl group, an amino acid residue, or a C_1-C_{17} alkyl group.

In the examination report of October 22, 2008, the Examiner has rejected all the claims for lack of enablement. As particularly stated by the Examiner:

(i) The claims are drawn to variables R^3 and R^4 that are independently H. The specification discloses compounds in which R^3 and R^4 are both simultaneously H. There is no guidance, direction or working example for making compounds wherein R^3 is H and R^4 is anything other than H. The specification does not disclose any prior art citation in lieu of enabling disclosure for obtaining such compounds.

(ii) The claims are drawn to compounds of formula I wherein R^2 is ammonium or a metal cation. Step 4 of working examples 1 and 2 allegedly describe the synthesis of sodium salt of 2-caryloyl and 2-palmitoyl ascorbic acid starting from the product of the step 3 which describes the <u>basification</u> of 5,6-isopropylidene ascorbic acid with sodium carbonate thus enabling the formation of R^2 sodium cation, that is compound of formula I wherein R^3 and R^4 are protected as isopropylidene ketal. The described reaction in step 4 involves treatment of the product of step 3 with methanolic aqueous HCL and washing with sodium chloride up to pH 7. The treatment of an isopropylidene compound with aqueous HCl acid is known in the art to deprotect the hydroxyl groups leading to the formation of R^3 and R^4. However, acid treatment of the sodium salt (R^2=Na) of an organic acid is expected to result in the neutralization of the salt resulting in the formation of R^2=H. Even though the specification discloses that the product is washed with sodium chloride to pH 7, it is art recognized fact that washing of organic acids (R^2=H) with sodium chloride cannot lead to salt formation.

(iii) There is no teaching in the specification on how to make aminoacid ester derivatives attachment, except for the generic statement that the amino group is protected prior to esterification. In addition, the disclosure in the specification is limited to speculation that the compounds are expected to be better than ascorbic acid; however, no data is presented.

In my opinion, and as demonstrated below by actual experiments, the Examiner is incorrect, because those skilled in the art would be able to practice the invention claimed based on what is disclosed in our above-identified patent application, coupled with common knowledge in the field.

The Examiner has further rejected the claims as being obvious over Shimizu *et al.* (EP 0619313) and Strelchler *et al.* (US 6,143,906), independently. According to the Examiner, Shimizu *et al.* teaches compounds of the instant formula wherein R^2 is lithium; according to the Examiner, however, it would be obvious to replace the lithium ion with other metal ions as defined in the present application for optimization of physical and chemical properties. Strelchler *et al.* discloses compounds of the instant formula wherein R^1 is C_6 acyl group; however, it would be obvious, according to the Examiner, to replace the C_6 acyl group with a homologous group.

By tests we have conducted, which are reproduced below, we have shown that the compounds of the present invention possess a surprisingly improved stability compared with that of ascorbic acid, making these compounds less sensitive to oxidative compounds such as free radicals. This activity is surprising, as it would not have been reasonably expected from what is known in the prior literature.

Attached herein below are the description and the results of various experiments recently conducted under my supervision in which examples 1 and 2 describe the complete synthesis of the ascorbic acid derivatives specifically described in the examples of the present application, i.e., the sodium salts of 2-capryloyl ascorbic acid (**DVC-12**) and of 2-palmitoyl ascorbic acid (**DVC-16**), including specific data regarding the process, yields, purity and spectroscopic data; and examples 3 and 4 describe antioxidant properties and stability studies, respectively, comparing the aforesaid derivatives with ascorbic acid.

EXAMPLES

Example 1. Synthesis of sodium salt of 2-capryloyl ascorbic acid (DVC-12)

(i) Synthesis of 5,6-isopropylidenyl ascorbic acid

20 g (0.125 mol) of anhydrous cupric sulfate were added to a suspension of 20 g (0.114 mol) of ascorbic acid in 660 ml of dry acetone. The reaction mixture was stirred for 20 h at room temperature and the process was monitored by TLC ($CHCl_3$-MeOH-H_2O, 10:10:3). The reaction mixture was filtered, the filtrate was evaporated, and the obtained residue was dried under reduced pressure to afford 5,6-isopropylidenyl ascorbic acid (22.57 g, 92%) as white solid. Analytical

sample was obtained by crystallization from $CHCl_3/MeOH$, R_f 0.51 ($MeOH-CHCl_3$, 2:3). ^1H NMR (500 MHz, MeOD-d_4), δ_H: 1.21 [c, 3H, $C(CH_3)_2$], 1.25 [c, 3H, $C(CH_3)_2$], 3.95 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6a}$ = 6.5 Hz, H-6a), 4.08 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6b}$ = 7.1 Hz, H-6b), 4.23 (ddd, 1H, $J_{4,5}$ = 2.7 Hz, $J_{5,6a}$ = 6.5 Hz, $J_{5,6b}$ = 7.1 Hz, H-5), 4.61 (d, 1H, $J_{4,5}$ = 2.7 Hz, H-4). ^{13}C NMR (125 MHz, MeOD-d_4), δ_C: 24.5, 25.0 [(isopropylidene, $C(CH_3)_2$], 65.3 (C-6), 74.2 (C-5), 75.6 (C-4), 109.9 (C-8), 118.8 (C-2), 153.1 (C-3), 172.0 (C-1). MALDI TOF MS calculated for $C_9H_{12}O_6$ ($[M + Na]^+$) *m/e* 239.2, measured *m/e* 239.4.

(ii) Synthesis of 2-capryloyl-5,6-isopropylidenyl ascorbic acid

Capryloyl chloride (10.30 g, 0.063 mol) was added dropwise at 0°C to a solution of 5,6-isopropylidenyl ascorbic acid (13.0 g, 0.060 mol) in dry pyridine (120 ml). The reaction system was stirred for 1.5 h at 0°C and the process was monitored by TLC ($CHCl_3$-MeOH, 3:1). Ice water (300 ml) was then added and the reaction mixture was adjusted to pH 3 using phosphoric acid (~10 ml) and extracted with ethyl acetate (2x100 ml). Combined extracts were washed with a saturated solution of sodium chloride up to pH 7. The washed organic layer was dried with anhydrous $MgSO_4$ and concentrated by vacuum. The residue was washed with hexane and concentrated by vacuum to give 18.97 g (92%) of 2-capryloyl-5,6-isopropylidenyl ascorbic acid as white solid. Analytical sample was obtained by crystallization from EtOAc/hexane, R_f 0.38 ($MeOH-CHCl_3$, 1:4). ^1H NMR (500 MHz, $CDCl_3$), δ_H: 0.88 [(t, CH_3, 3H, C(O) $(CH_2)_6CH_3$), 1.32 (m, $4CH_2$, 8H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.36 [(c, 3H, $C(CH_3)_2$), 1.39 [c, 3H, $C(CH_3)_2$], 1.70 (t, CH_2, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 2.59 [(t, CH_2, 2H, $C(O)CH_2(CH_2)_5 CH_3$), 4.09 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6a}$ = 6.4 Hz, H-6a), 4.19 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6b}$ = 7.0 Hz, H-6b), 4.43 (ddd, 1H, $J_{4,5}$ = 2.5 Hz, $J_{5,6a}$ = 6.4 Hz, $J_{5,6b}$ = 7.0 Hz, H-5), 4.69 (d, 1H, $J_{4,5}$ = 2.5 Hz, H-4). ^{13}C NMR (125 MHz, $CDCl_3$), δ_C: 15.8 [$C(O)(CH_2)_6CH_3$], 24.3, 26.3, 30.5, 30.7, 34.3, 35.5 ($C(O)(CH_2)_6CH_3$), 27.3, 27.9 [(isopropylidene, $C(CH_3)_2$], 67.1 (C-6), 75.2 (C-5), 76.4 (C-4), 112.5 (C-8), 117.1 (C-2), 157.0 (C-3), 168.2 (C-1), 176.0 ($C(O)(CH_2)_6CH_3$). MALDI TOF MS calculated for $C_{17}H_{26}O_7$ ($[M + Na]^+$) *m/e* 365.2, measured *m/e* 365.1.

(iii) Synthesis of sodium salt of 2-capryloyl-5,6-isopropylidenyl ascorbic acid

3.0 g of 2-capryloyl-5,6-isopropylidenyl ascorbic acid were dissolved in 150 ml ethyl acetate and put into a separated funnel, and a solution of sodium carbonate (3 M, 50 ml) was then added to the prepared solution. After mixing and exposing for about 10 min, a triple-phase system

was obtained. The intermediate phase was selected, filtered and concentrated. The yield of the product equals 65-70%. The sodium salt of 2-capryloyl-5,6-isopropylidenyl ascorbic acid was obtained as light-yellow solid amorphous substance. Analytical sample was obtained by crystallization from MeOH, R_f 0.41 (MeOH-$CHCl_3$, 1:3). ^1H NMR (500 MHz, D_2O), δ_H: 0.79 [(t, CH_3, 3H, C(O)$(CH_2)_6CH_3$), 1.22 (m, $4CH_2$, 8H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 1.30 [(c, 3H, $C(CH_3)_2$)], 1.33 [c, 3H, $C(CH_3)_2$], 1.61 (t, CH_2, 2H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 2.48 [(t, CH_2, 2H, C(O)$CH_2(CH_2)_5CH_3$), 4.07 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6a}$ = 6.4 Hz, H-6a), 4.21 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6b}$ = 7.0 Hz, H-6b), 4.45 (ddd, 1H, $J_{4,5}$ = 2.5 Hz, $J_{5,6a}$ = 6.4 Hz, $J_{5,6b}$ = 7.0 Hz, H-5), 4.69 (d, 1H, $J_{4,5}$ = 2.5 Hz, H-4). ^{13}C NMR (125 MHz, D_2O), δ_C: 15.2 [C(O)$(CH_2)_6CH_3$], 23.8, 26.3, 29.8, 30.1, 32.9, 34.8 (C(O)$(CH_2)_6CH_3$), 26.1, 26.7 [(isopropylidene, $C(CH_3)_2$], 66.9 (C-6), 75.7 (C-5), 78.6 (C-4), 112.5 (C-8), 111.2 (C-2), 167.0 (C-1), 177.0 (C-3), 180.5 (C(O)$(CH_2)_6CH_3$). MALDI TOF MS calculated for $C_{17}H_{25}NaO_7$ ($[M + Na]^+$) *m/e* 387.1, measured *m/e* 387.0.

(iv) Synthesis of 2-capryloyl ascorbic acid

2-capryloyl ascorbic acid was obtained by deprotection of the 5- and 6-hydroxyl groups of 2-capryloyl-5,6-isopropylidenyl ascorbic acid (3.0 g, 0.0088 mol) from step *(ii)* above, at mild conditions, by means of the reaction mixture MeOH : H_2O : 2N HCl = 30:2:1 (v/v/v) at 4°C for 24 h. The process was monitored by TLC (MeOH/$CHCl_3$, 2:3). The reaction mixture was evaporated and the residue was washed with methanol (3x50 ml) with following evaporation. Then, the residue was dissolved in 5 ml of methanol, diluted with EtOAc (100 ml), washed with a saturated solution of sodium chloride up to pH 7, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (silica gel, MeOH/$CHCl_3$, 2:3) to afford 2-capryloyl ascorbic acid as a white solid (2.43 g, 92%). Analytical sample was obtained by crystallization from $CHCl_3$/MeOH, R_f 0.54 (MeOH-$CHCl_3$, 2:3). ^1H NMR (500 MHz, $CHCl_3$/MeOD-d_4), δ_H: 0.82 [(t, CH_3, 3H, C(O) $(CH_2)_6CH_3$), 1.29 (m, $4CH_2$, 8H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 1.58 (t, CH_2, 2H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 2.53 [(t, CH_2, 2H, C(O)$CH_2(CH_2)_5CH_3$), 4.11 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6a}$ = 6.4 Hz, H-6a), 4.26 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6b}$ = 7.0 Hz, H-6b), 4.49 (ddd, 1H, $J_{4,5}$ = 2.5 Hz, $J_{5,6a}$ = 6.4 Hz, $J_{5,6b}$ = 7.0 Hz, H-5), 4.73 (d, 1H, $J_{4,5}$ = 2.5 Hz, H-4). ^{13}C NMR (125 MHz, $CHCl_3$/MeOD-d_4), δ_C: 15.6 [C(O)$(CH_2)_6CH_3$], 24.1, 26.8, 30.4, 31.2, 33.7, 35.3 (C(O)$(CH_2)_6CH_3$), 67.8 (C-6), 76.9 (C-5), 79.8 (C-4), 114.0 (C-2), 169.8 (C-1), 178.5 (C-3), 181.2 (C(O)$(CH_2)_6CH_3$). MALDI TOF MS calculated for $C_{14}H_{22}O_7$ ($[M + K]^+$) *m/e* 341.1, measured *m/e* 341.4.

(v) Synthesis of sodium salt of 2-capryloyl ascorbic acid (DVC-12)

DVC-12, illustrated in **Scheme 1** hereinbelow, was obtained by treatment of 2-capryloyl ascorbic acid from step (*iv*) above, with water solution of sodium carbonate. 2-Capryloyl ascorbic acid (2.0 g, 0.0066 mol) was suspended in water (45 ml) at 4°C, sodium carbonate (0.35 g, 0.0033 mol) in 5 ml of water was added dropwise, and the reaction mixture was stirred for 20 min. Then, ethanol (15 ml) was added to obtain a fully transparent solution. The reaction mixture was allowed to rise to room temperature, stirred for 30 min and evaporated. The residue was washed with ethanol (2x50 ml) and then with acetone (3x50 ml), with following evaporation, and was dried under reduced pressure to afford light-yellow solid (1.75 g, 81%). Analytical sample was obtained by crystallization from MeOH, R_f 0.51 ($MeOH/CHCl_3$, 1:3). ^1H NMR (500 MHz, MeOD-d_4/D_2O), δ_H: 0.85 [(t, CH_3, 3H, C(O) $(CH_2)_6CH_3$), 1.26 (m, $4CH_2$, 8H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.53 (t, CH_2, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 2.51 [(t, CH_2, 2H, $C(O)CH_2(CH_2)_5$ CH_3), 4.07 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6a}$ = 6.4 Hz, H-6a), 4.20 (dd, 1H, $J_{6a,6b}$ = 8.7 Hz, $J_{5,6b}$ = 7.0 Hz, H-6b), 4.43 (ddd, 1H, $J_{4,5}$ = 2.5 Hz, $J_{5,6a}$ = 6.4 Hz, $J_{5,6b}$ = 7.0 Hz, H-5), 4.66 (d, 1H, $J_{4,5}$ = 2.5 Hz, H-4). ^{13}C NMR (125 MHz, MeOD-d_4/D_2O), δ_C: 15.6 [$C(O)(CH_2)_6CH_3$], 24.9, 26.0, 30.1, 31.8, 34.3, 36.2 ($C(O)(CH_2)_6CH_3$), 67.1 (C-6), 77.3 (C-5), 79.0 (C-4), 113.6 (C-2), 167.4 (C-1), 179.0 (C-3), 182.6 ($C(O)(CH_2)_6CH_3$). MALDI TOF MS calculated for $C_{14}H_{21}NaO_7$ ($[M + Na]^+$) *m/e* 347.1, measured *m/e* 347.2.

Example 2. Synthesis of sodium salt of 2-palmitoyl ascorbic acid (DVC-16)

(i) Synthesis of 5,6-isopropylidenyl ascorbic acid

5,6-isopropylidenyl ascorbic acid was synthesized as described in step (*i*) of example 1 above.

(ii) Synthesis of 2-palmitoyl-5,6-isopropylidenyl ascorbic acid

Palmitoyl chloride (17.58 g, 0.064 mol) was added dropwise at 0°C to a solution of 5,6-isopropylidenyl ascorbic acid (13.17 g, 0.061 mol) in dry pyridine (110 ml). The reaction system was stirred for 1.5 h at 0°C and the process was monitored by TLC (chloroform-methanol, 3:1). Ice water (300 ml) was then added and the reaction mixture was adjusted to pH 3 using phosphoric acid (~10 ml) and extracted with ethyl acetate (2x100 ml). Combined extracts were washed with saturated solution of sodium chloride up to pH 7. The washed organic layer was dried with anhydrous $MgSO_4$ and concentrated by vacuum. The residue was washed with hexane and was then

concentrated by vacuum to give 26.27 g (95%) of 2-palmitoyl-5,6-isopropylidene ascorbic acid. Analytical sample was obtained by crystallization from EtOAc/hexane, R_f 0.45 (MeOH-$CHCl_3$, 1:4). ^{1}H NMR (500 MHz, $CDCl_3$), δ_H: 0.80 [(t, CH_3, 3H, $C(O)(CH_2)_{14}CH_3$), 1.23 (m, 12CH_2, 24H, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.36 [(c, 3H, $C(CH_3)_2$)], 1.39 [c, 3H, $C(CH_3)_2$], 1.61 (t, CH_2, 2H, $C(O)(CH_2)(CH_2)(CH_2)_{12}CH_3$), 2.45 [(t, CH_2, 2H, $C(O)CH_2(CH_2)_{13}CH_3$), 4.09 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6a}$ = 6.5 Hz, H-6a), 4.19 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6b}$ = 7.2 Hz, H-6b), 4.43 (ddd, 1H, $J_{4,5}$ = 2.3 Hz, $J_{5,6a}$ = 6.5 Hz, $J_{5,6b}$ = 7.2 Hz, H-5), 4.69 (d, 1H, $J_{4,5}$ = 2.3 Hz, H-4). ^{13}C NMR (125 MHz, $CDCl_3$), δ_C: 13.8 [$C(O)(CH_2)_{14}CH_3$], 22.5, 24.3, 28.8, 29.1, 29.2, 29.4, 29.5, 31.7, 33.1, 33.9 ($C(O)(CH_2)_{14}CH_3$), 25.2, 25.5 [(isopropylidene, $C(CH_3)_2$], 65.1 (C-6), 74.4 (C-5), 76.7 (C-4), 113.6 (C-8), 117.1 (C-2), 160.9 (C-3), 168.1 (C-1), 171.4 ($C(O)$ $(CH_2)_{14}CH_3$). MALDI TOF MS calculated for $C_{25}H_{42}O_7$ ($[M + Na]^+$) *m/e* 477.2, measured *m/e* 477.4.

(iii) Synthesis of sodium salt of 2-palmitoyl-5,6-isopropylidenyl ascorbic acid

3.0 g of 2-palmitoyl-5,6-isopropylidenyl ascorbic acid were dissolved in 150 ml of ethyl acetate and put into the separated funnel, and a solution of sodium carbonate (3 M, 50 ml) was then added to the prepared solution. After mixing and exposing for about 10 min, the triple-phase system was obtained. The intermediate phase was selected, filtered and concentrated. The yield of the final product equals 65-70%. The sodium salt of 2-palmitoyl-5,6-isopropylidenyl ascorbic acid was obtained as light-yellow solid amorphous substance. Analytical sample was obtained by crystallization from MeOH/$CHCl_3$, R_f 0.42 (MeOH-$CHCl_3$, 1:5). ^{1}H NMR (500 MHz, D_2O), δ_H: 0.67 [(t, CH_3, 3H, $C(O)(CH_2)_{14}CH_3$), 1.17 (m, 12CH_2, 24H, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.18 [(c, 3H, $C(CH_3)_2$)], 1.20 [c, 3H, $C(CH_3)_2$], 1.46 (t, CH_2, 2H, $C(O)(CH_2)(CH_2)(CH_2)_{12}CH_3$), 2.32 [(t, CH_2, 2H, $C(O)CH_2(CH_2)_{13}CH_3$), 3.92 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6a}$ = 6.5 Hz, H-6a), 4.02 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6b}$ = 7.2 Hz, H-6b), 4.21 (ddd, 1H, $J_{4,5}$ = 2.3 Hz, $J_{5,6a}$ = 6.5 Hz, $J_{5,6b}$ = 7.2 Hz, H-5), 4.27 (d, 1H, $J_{4,5}$ = 2.3 Hz, H-4). ^{13}C NMR (125 MHz, D_2O), δ_C: 13.1 [$C(O)(CH_2)_{14}CH_3$], 22.1, 24.0, 28.2, 28.9, 29.1, 29.3, 29.5, 31.5, 32.6, 32.8 ($C(O)(CH_2)_{14}CH_3$), 24.3, 24.7 [(isopropylidene, $C(CH_3)_2$], 64.7 (C-6), 73.5 (C-5), 76.0 (C-4), 112.9 (C-8), 115.8 (C-2), 158.4 (C-3), 166.2 (C-1), 170.7 ($C(O)(CH_2)_{14}CH_3$). MALDI TOF MS calculated for $C_{25}H_{41}NaO_7$ ($[M + Na]^+$) *m/e* 499.3, measured *m/e* 499.1.

(iv) Synthesis of 2-palmitoyl ascorbic acid

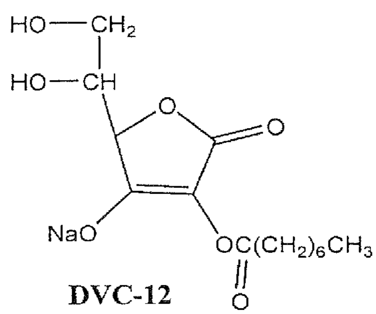
2-palmitoyl ascorbic acid was obtained by deprotection of the 5- and 6-hydroxyl groups of 2-palmitoyl-5,6-isopropylidenyl ascorbic acid (3.0 g, 0.0066 mol) from step (*ii*), at mild conditions, by means of the reaction mixture MeOH : H_2O : 2N HCl = 30:2:1 (v/v/v) at 4°C for 24 h. The process was monitored by TLC ($MeOH/CHCl_3$, 3:7). The reaction mixture was evaporated and the residue was washed with methanol (3x50 ml) with following evaporation. Then, the residue was dissolved in 5 ml of methanol, diluted with EtOAc (100 ml), washed with a saturated solution of sodium chloride up to pH 7, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (silica gel, $MeOH/CHCl_3$, 3:7) to afford 2-palmitoyl ascorbic acid as white solid (2.57 g, 94%). Analytical sample was obtained by crystallization from $CHCl_3$/MeOH, R_f 0.38 ($MeOH-CHCl_3$, 3:7). ^{1}H NMR (500 MHz, $CHCl_3$/MeOD-d_4), δ_H: 0.73 [(t, CH_3, 3H, $C(O)(CH_2)_{14}CH_3$), 1.27 (m, $12CH_2$, 24H, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.56 (t, CH_2, 2H, $C(O)(CH_2)(CH_2)(CH_2)_{12}CH_3$), 2.41 [(t, CH_2, 2H, $C(O)CH_2(CH_2)_{13}CH_3$), 4.02 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6a}$ = 6.5 Hz, H-6a), 4.13 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6b}$ = 7.2 Hz, H-6b), 4.30 (ddd, 1H, $J_{4,5}$ = 2.3 Hz, $J_{5,6a}$ = 6.5 Hz, $J_{5,6b}$ = 7.2 Hz, H-5), 4.41 (d, 1H, $J_{4,5}$ = 2.3 Hz, H-4). ^{13}C NMR (125 MHz, $CHCl_3$/MeOD-d_4), δ_C: 14.8 [$C(O)(CH_2)_{14}CH_3$], 22.9, 24.4, 28.7, 29.6, 29.8, 29.3, 29.9, 31.9, 32.5, 33.4 ($C(O)(CH_2)_{14}CH_3$), 66.1 (C-6), 74.3 (C-5), 76.9 (C-4), 117.0 (C-2), 161.2 (C-3), 168.7 (C-1), 172.5 ($C(O)(CH_2)_{14}CH_3$). MALDI TOF MS calculated for $C_{22}H_{38}O_7$ ($[M + K]^+$) *m/e* 453.3, measured *m/e* 453.5.

(v) Synthesis of sodium salt of 2-palmitoyl ascorbic acid (DVC-16)

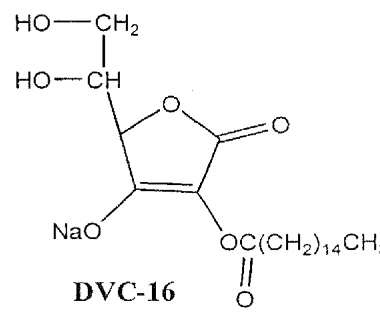
DVC-16, illustrated in **Scheme 1** hereinbelow, was obtained by treatment of 2-palmitoyl ascorbic acid from step (*iv*) with water solution of sodium carbonate. In particular, 2-Palmitoyl ascorbic acid (2.3 g, 0.0056 mol) was suspended in water (45 ml) at 4°C, sodium carbonate (0.30 g, 0.0028 mol) in 5 ml of water was added dropwise, and the reaction mixture was stirred for 20 min. The reaction mixture was allowed to rise to room temperature, stirred for 30 min and was then filtered and lyophilized to give sodium salt of 2-palmitoyl ascorbic acid as white foamy solid (2.15 g, 89%). Analytical sample was obtained by crystallization from $MeOH/H_2O$, R_f 0.57 ($MeOH/CHCl_3$, 1:3). ^{1}H NMR (500 MHz, MeOD-d_4/D_2O), δ_H: 0.79 [(t, CH_3, 3H, $C(O)(CH_2)_{14}CH_3$), 1.29 (m, $12CH_2$, 24H, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.61 (t, CH_2, 2H, $C(O)(CH_2)(CH_2)(CH_2)_{12}CH_3$), 2.41 [(t, CH_2, 2H, $C(O)CH_2(CH_2)_{13}CH_3$), 4.09 (dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6a}$ = 6.5 Hz, H-6a), 4.11

(dd, 1H, $J_{6a,6b}$ = 8.9 Hz, $J_{5,6b}$ = 7.2 Hz, H-6b), 4.35 (ddd, 1H, $J_{4,5}$ = 2.3 Hz, $J_{5,6a}$ = 6.5 Hz, $J_{5.6b}$ = 7.2 Hz, H-5), 4.49 (d, 1H, $J_{4,5}$=2.3 Hz, H-4). ^{13}C NMR (125 MHz, MeOD-d_4/D_2O), δ_C: 14.8 [C(O)$(CH_2)_{14}$*C*H_3], 23.3, 24.9, 29.4, 29.9, 30.2, 30.4, 30.6, 32.5, 32.99, 33.8 (C(O)$(CH_2)_{14}CH_3$), 66.9 (C-6), 74.3 (C-5), 77.3 (C-4), 117.8 (C-2), 162.0 (C-3), 169.6 (C-1), 172.9 (*C*(O)$(CH_2)_{14}CH_3$). MALDI TOF MS calculated for $C_{22}H_{37}NaO_7$ ([M + K]$^+$) *m/e* 475.2, measured *m/e* 475.0.

Scheme 1: Chemical structures of DVC-12 and DVC-16



DVC-12



DVC-16

The above synthesis examples show that the compounds of the present invention can be routinely made by those skilled in the art based on what is disclosed in our above-identified patent application, coupled with conventional knowledge in the field.

Example 3. Antioxidant properties of DVC-12 and DVC-16 *vs.* ascorbic acid

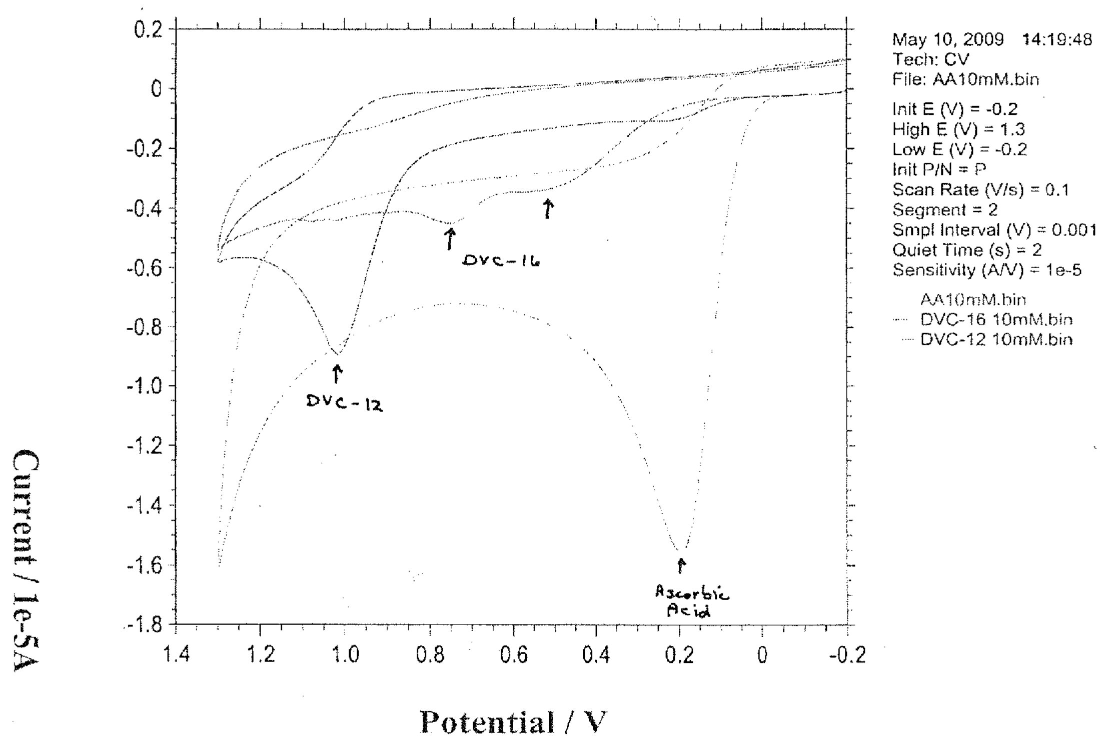
The antioxidant properties of ascorbic acid, **DVC-12** and **DVC-16** were evaluated by three different methods as described herein:

3.1 Cyclic voltammeteric measurements:

In this experiment, the overall reducing power of the various compounds, which correlates with the overall scavenging activity thereof, was evaluated as described in Kohen *et al.*, 1999 and 2000 (Kohen *et al.*, Evaluation of the overall low molecular weight antioxidant activity of biological fluids and tissues by cyclic voltammeter, In *Methods in Enzymology*, L. Packer (ed.), Academic Press 300, San Diego, CA, **1999**, 285-295; Kohen *et al.*, Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues, *Free Radic. Biol. Med.*, **2000**, 28, 871-879), and the cyclic voltammograms of **DVC-16**, **DVC-12** and ascorbic acid (Ref. Ag, $AgNO_3$, WE: glossy carbon, auxiliary electrode: Pt, scan rate 100 mV/sec) are presented

in **Fig. 1**. As shown in **Fig. 1**, ascorbic acid possessed peak potential at 0.28 V, **DVC-16** demonstrated 2 small anodic waves at ~0.45 V and ~0.75 V and **DVC-12** demonstrated one anodic wave at ~1 V. The results obtained indicate that ascorbic acid possesses thc strongest electron donating ability, i.e., the highest antioxidant properties. **DVC-16** is also capable of donating electron(s) and can thus bc considered as a reducing antioxidant although weaker then ascorbic acid (the lower the potential the compound has, the stronger its ability to donate electron(s)). An anodic wave of around 1 V, as obtained for **DVC-12**, suggests relatively weak reducing ability and thus relatively weak antioxidant properties.

Fig. 1

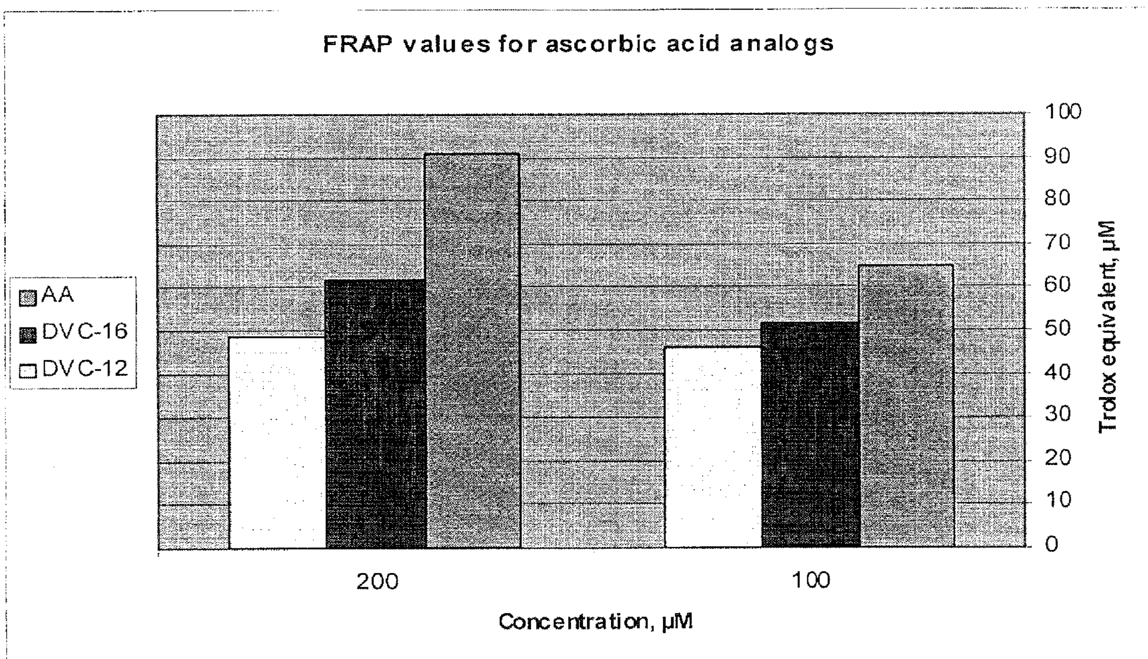


3.2 Reducing ability as indicated by the FRAP assay

In this experiment, the ferric reducing antioxidant power (FRAP) assay described in Benzie and Strain, 1999 (Benzie, I.F., Strain, J.J., Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration, *Methods in Enzymology*, **1999**, 299, 15-23) was used to evaluate the reducing ability of the three compounds, which are essential for the

antioxidant activity thereof, and the ability of the compounds to reduce ferric ions to ferrous is presented in **Fig. 2**. As shown in **Fig. 2,** in comparison to ascorbic acid, **DVC-16** in concentrations of 100 μM and 200μM showed reducing capacity of 79.6% and 68.2% relative to ascorbic acid, respectively, while **DVC-12** in concentrations 100 μM and 200 μM showed reducing capacity of 70.4% and 53.9% relative to ascorbic acid, respectively.

Fig. 2



3.3 Scavenging ability as evaluated by the ORAC assay:

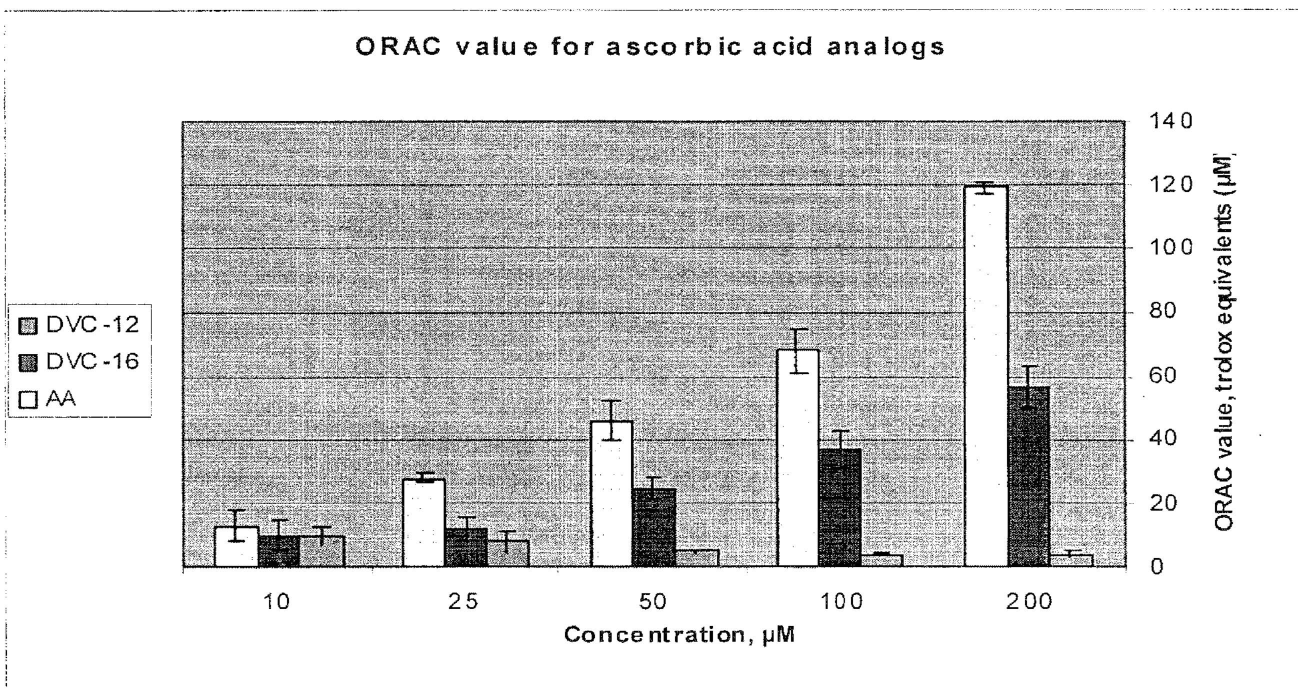
In this experiment, the oxygen-radical absorbing capacity (ORAC) assay described in Cao *et al.*, 1993 (Cao *et al.*, Oxygen-radical absorbance capacity assay for antioxidants, *Free Radic Biol Med*, **1993**, 14, 303-11) was used to evaluate the scavenging ability of the three compounds. Ascorbic acid, **DVC-16** and **DVC-12** were tested in 5 different concentrations, and the results are expressed as ORAC units, wherein 1 ORAC unit equals the net protection produced by 1 μM Trolox (a Hoffman-LaRoshe's trade name for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, which is a water-soluble derivative of vitamin E). The ORAC values for each one of the compounds tested are summarized in **Table 1** and are further presented in **Fig. 3**. As shown in **Fig. 3,** the oxygen radical scavenging capacity of **DVC-16** was significantly better than that of **DVC-12**, while ascorbic acid demonstrated the highest ability to scavenge peroxyl radicals. As particularly

shown, although **DVC-16** has a scavenging capacity which is about 50% of that of ascorbic acid, both ascorbic acid and **DVC-16** possess a dose dependent ability to scavenge peroxyl radicals and are thus able to prevent lipid peroxidation process.

Table 1: Oxygen radical scavenging capacity of **DVC-16**, **DVC-12** and ascorbic acid

μM	DVC-12	DVC-16	Ascorbic acid
200	4.35	56.57	119.30
100	3.99	37.00	67.80
50	5.20	24.48	45.95
25	7.94	11.94	27.66
10	9.19	9.96	12,72

Fig. 3

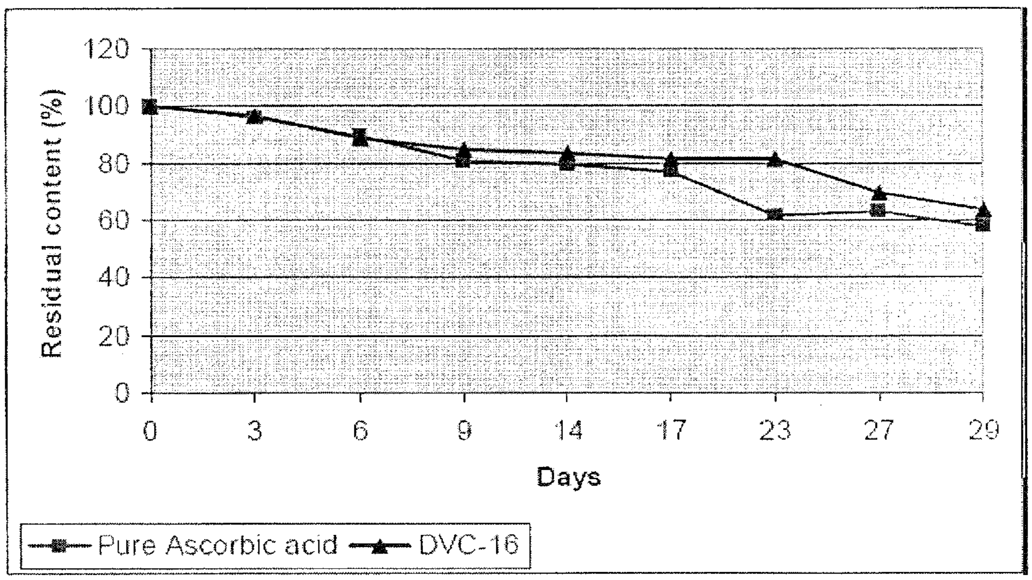


Example 4. Stability study of DVC-16 *vs.* ascorbic acid

4.1 Stability of DVC-16 in water at room temperature for long term

In this experiment, the stability of ascorbic acid and **DVC-16** 16 in water, at room temperature, was evaluated. In particular, ascorbic acid and its derivative **DVC-16** (1% each) were incubated in water (pH 6.7) at room temperature, and the residual contents were measured by HPLC. The results presented in **Fig. 4** show that there was a gradual reduction in the residual content of both ascorbic acid and its analog **DVC-16** over time, wherein **DVC-16** was slightly more stable. These results demonstrate that the stability of **DVC-16** under these conditions is at least not any lesser than that of ascorbic acid and may be even considered slightly better that that of ascorbic acid.

Fig. 4

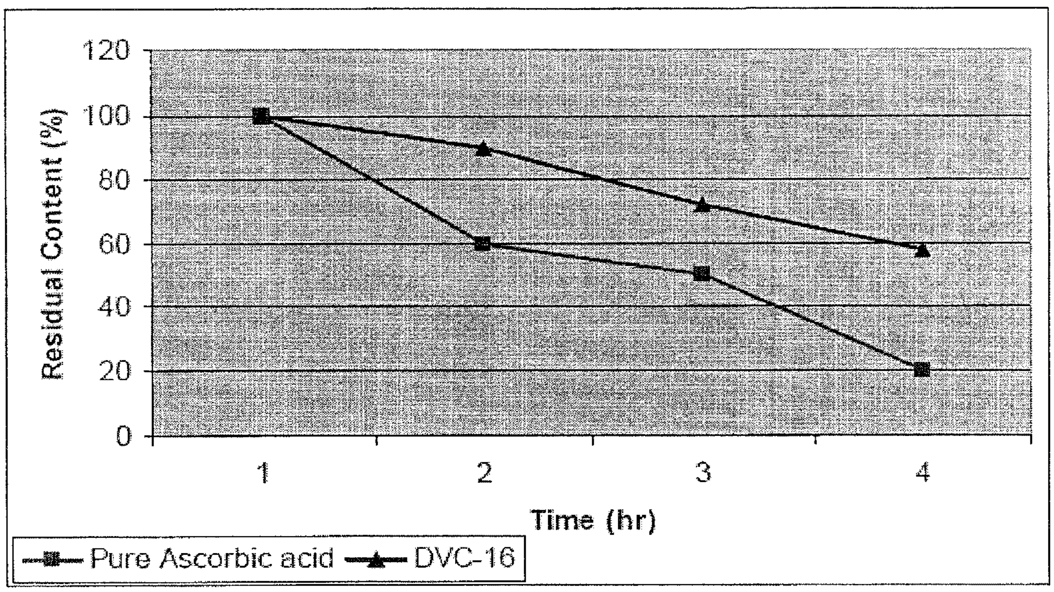


4.2 Stability of DVC-16 in water at 50°C for short term

In this experiment, the stability of ascorbic acid and **DVC-16** in water, at 50°C, was evaluated. In particular, ascorbic acid and its derivative **DVC-16** (50 μM each) were incubated in water (pH 7.0) at 50°C, and the residual contents were measured by HPLC. The results presented in **Fig. 5** show that after 4 hours of incubation, the residual content of ascorbic acid was reduced to 20% of the original content only, whereas the residual content of **DVC-16** was reduced to

approximately 60% the original content. These results demonstrate that under the tested conditions, the stability of **DVC-16** was remarkably higher than that of ascorbic acid, wherein the residual content of DVC-16 following 4 hours of incubation was three times that of ascorbic acid, and further indicate that DVC-16 is expected to be significantly more stable than ascorbic acid under physiological conditions as well.

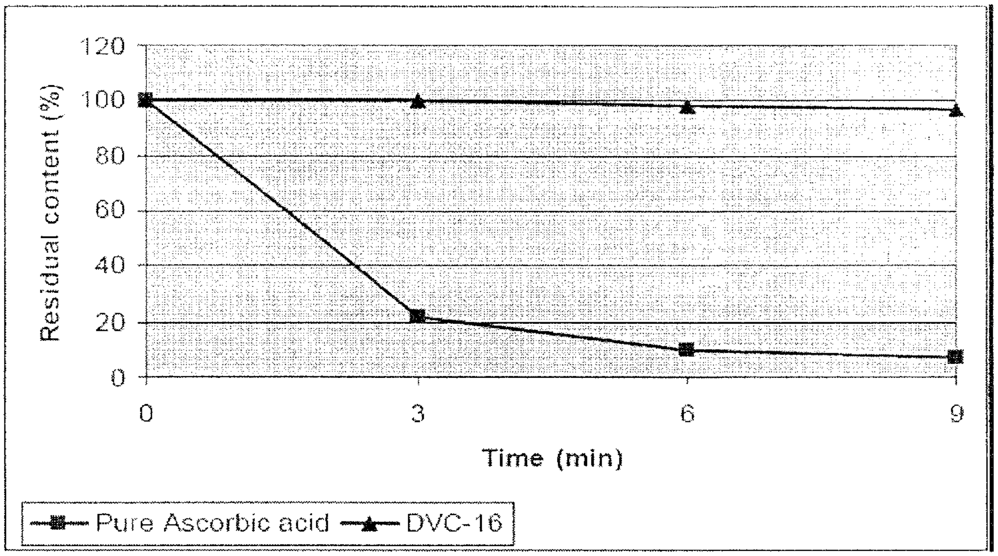
Fig. 5



4.3 Stability of DVC-16 under oxidative conditions

In this experiment, the stability of ascorbic acid and its derivative **DVC-16** under oxidative conditions was evaluated. In particular, ascorbic acid and **DVC-16** (100 μM each) were incubated in a solution containing $CuSO_4$ (20 μM) at room temperature, and the residual contents of each one of these compounds were measured by HPLC. The results presented in **Fig. 5** show that while the residual content of ascorbic acid dropped to about 20% after 3 minutes of incubation and then to about 10% or less after 6 minutes of incubation, the residual content of **DVC-16** remained close to 100% throughout the whole experiment, indicating that contrary to ascorbic acid, **DVC-16** is highly stable under oxidative conditions.

Fig. 6



Discussion

As demonstrated in examples 1 and 2 above, contrary to the Examiner's contention, compounds as defined in the present application and, in particular, the compounds specifically described in the present description, i.e., the derivatives **DVC-16** and **DVC-12**, can be obtained by any person skilled in the art using processes which were almost entirely disclosed in the present specification.

It is worth noting that in the same way as described in examples 1 and 2 of this declaration, other compounds of the formula defined in claim 1 of the present application may be obtained, using various technologies and procedures that have all been described in the literature and are known to any person skilled in the art.

Such compounds may be, for example, ascorbic acid derivatives of the formula defined in claim 1, in which the metal in position 2 is different than sodium and/or the esters at position 1 are derived from other carboxylic acids, preferably fatty acids such as, without limitation, capric, undecanoic, lauric, tridecanoic, myristic, pentadecanoic or stearic acid, or from amino acids such as glycine or alanine.

The Examiner's assertion according to which the present specification does not teach the preparation of other compounds of the present application, wherein R^3 is H and R^4 is anything other

than H, or the opposite, is indeed correct; however, such compounds may be obtained by any person skilled in the art using various synthesis methods and procedures available in the art, for example, the procedure disclosed in Strelchler *et al.*, cited by the Examiner, for the preparation of 6-O-palmitoyl-2-O-sorbyl-L-ascorbic acid.

As may be concluded from the experiments described in example 3 of this declaration, the ascorbic acid derivatives **DVC-12** and **DVC-16** possess different levels of antioxidant activity, which are lower than that of ascorbic acid. Nevertheless, the reduced antioxidant activity of these compounds is most probably the reason for their stability that is significantly higher than that of ascorbic acid. In fact, as postulated by the inventors of the present application at the time this application was filed, the lower activity of the compounds of the present application is directly correlated with the increased stability thereof, i.e., the fact that these compounds have an antioxidant activity that is generally lower than that of ascorbic acid makes them less sensitive to oxidative compounds such as free radicals, thus enable them to be actively available for a significantly longer period of time, as indeed clearly shown in example 4 of this declaration.

As particularly shown in example 4 above, **DVC-16** was consistently more stable than ascorbic acid, and specifically under oxidative conditions. These results reinforce the hypothesis raised by the inventors at the time the invention was made and further indicate that by using the compounds of the present application we can benefit from their antioxidant activity for a longer period of time which compensates for the fact that they are not as potent antioxidant as free ascorbic acid (which, practically, can not be used due to its instability.)

The only compound exemplified in Shimizu *et al.*, cited by the Examiner, is 2-O-octadecylascorbic acid lithium, which is a compound similar to that of the formula defined in claim 1 of the present application, wherein R^1 is a C_{18} alkyl and R^2 is lithium. The compound disclosed by Shimizu *et al.* is not covered by the definition in claim 1. Furthermore, Shimizu *et al.* disclose this compound for preventing or treating a functional disorder of the circulatory system or cancer; however, it does no disclose nor suggests that this compound is more stable than ascorbic acid, which is the most important property of the compounds of the present application as clearly stated in the specification on page 7 lines 20-23. In view of that, the compounds of the present application cannot be considered as being obvious over Shimizu *et al.*

Strelchler *et al.*, cited by the Examiner, discloses the synthesis of four ascorbic acid derivatives, in particular, (i) 2,5,6-O-trisorbyl-L-ascorbic acid; (ii) 5,6-O-isopropylidene-2-O-

sorbyl-L-ascorbic acid; (iii) 2-O-sorbyl-L-ascorbic acid; and (iv) 6-O-palmitoyl-2-O-sorbyl-L-ascorbic acid, asserting that these derivatives are more stable than ascorbic acid. However, no concrete data is provided in this reference concerning either the activity or the stability of these derivatives, and a protocol for evaluating these parameters is not even suggested (it should be noted that the compounds disclosed in Strelchler *et al.* are not available, and therefore any evaluation thereof could not have been performed). In view of that, and as the properties of the compounds of the present application are clearly exemplified above, the latter cannot be considered as being obvious over the compounds of Strelchler *et al.* in my opinion.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

August 30, 2009
Date

Dr. Valery Belakhov

TAG RA-003 US

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August 2009

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PERSONAL DATA

Born:	February 17, 1957, Saint-Petersburg, Russia.
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Immigration to Israel:	November 16, 1990.

EDUCATION

1974-1979: Department of Chemical Engineering and Biotechnology, Saint-Petersburg State Chemical Pharmaceutical Academy, Saint-Petersburg, Russia.

1979: **M.Sc., Chemical Engineering,** Honors Degree.

1981-1984: Post-graduate Course at the Department of Organic Chemistry, Saint-Petersburg State Technological Institute (Technical University), Saint-Petersburg, Russia.

1984: **Ph.D., Chemistry.**

EMPLOYMENT

2001-present: **Senior Research Scientist & Depute Head of the Edith and Joseph Fisher Enzyme Inhibitors Laboratory**, Schulich Faculty of Chemistry, Technion - Israel Institute of Technology, Haifa, Israel.

1995-2001: **Senior Research Associate,** Department of Chemistry, Technion - Israel Institute of Technology, Haifa, Israel.

1991-1995: **Research Associate,** Department of Chemistry, Technion - Israel Institute of Technology, Haifa, Israel.

1987-1990: **Senior Research Scientist & Head of Research Group**, Department of Drug Standardization, Institute of Antibiotics and Medical Enzymes, Saint-Petersburg, Russia.

1984-1987: **Research Scientist**, Department of Technology of Antibiotics andNucleosides, Institute of Antibiotics and Medical Enzymes, Saint-Petersburg, Russia.

1979-1981: **Chemist-Engineer,** Department of Ready Drugs, Institute of Antibiotics and Medical Enzymes, Saint-Petersburg, Russia.

SCIENTIFIC DIRECTIONS

♦ ***Synthetic Organic Chemistry:*** Carbohydrate Chemistry, Chemistry of Organophosphorus and Organofluorine Compounds.

♦ ***Structure-Function Studies of the Crystal Structure of Enzymes:*** Synthesis of Mechanism-Based Inhibitors of KDO8P Synthase.

♦ ***Rational Design and Searh of New Derivatives of Aminoglycoside Antibiotics:*** Synthesis of Semi-Synthetic Derivatives of Aminoglycoside Antibiotics Targeting rRNA and Resistance-causing Enzymes.

♦ ***Chemical Modification of Polyene Macrolide Antibiotics:*** Search of Novel High-Effective Derivatives of Antifungal Polyene Macrolide Antibiotics.

♦ ***Chemical Engineering and Biotechnology:*** Biosynthesis, Purification and Characterization of Enzymes.

♦ ***Pesticide Chemistry:*** Application of Functionally Substituted Derivatives of Various Sugars as the Potential Herbicides and Fungicides.

SCIENTIFIC AND PROFESSIONAL PUBLICATIONS

My Full List of Scientific and Professional Publications includes 100 Original Papers in Journals, Papers in Books and Reviews, and 5 Patents, and about 200 Scientific Report Abstracts Published in Proceedings of National and International Conferences, Symposiums and Congresses.

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3. M. Fridman, **V. Belakhov**, Lac V. Lee, Fu-Sen Liang, C.H. Wong, T. Baasov, Dual Effect of Synthetic Aminoglycosides: Antibacterial Activity against *Bacillus anthracis* and Inhibition of Anthrax Lethal Factor, *Angewandte Chemie, Int. Ed. Eng.*, **2005**, 44(3), 447-452

4. C.M. Furdui, A.K. Sau, O. Yaniv, **V. Belakhov**, R.W. Woodard, T. Baasov, K.S. Anderson, The Use of (*E*)- and (*Z*)-Phosphoenol-3-fluoropyruvate as Mechanistic Probes Reveals Significant Differences Between the Active Sites of KDO8P and DAHP Synthases, *Biochemistry*, **2005**, 44(19), 7326-7335

5. R. Vainer, **V. Belakhov**, E. Rabkin, A. Sau, C. Furdui, K.S. Anderson, T. Baasov, N. Adir, Crystal Structures of *Escherichia coli* KDO8P Synthase Complexes Reveal the Source of Catalytic Irreversibility, *Journal of Molecular Biology*, **2005**, 351(3), 641-652

6. M. Hainrichson, V. Pokrovskaya, D. Shallom, M. Fridman, **V. Belakhov**, D. Shachar, S. Yaron, T. Baasov, Branched Aminoglycosides: Biochemical Studies and Antibacterial Activity of Neomycin B Derivatives, *Bioorganic & Medicinal Chemistry*, **2005**, 13(20), 5797-5807

7. **V. Belakhov**, Yu.D. Shenin, Synthesis and Antifungal Activity of N-Benzyl Derivatives of Amphotericin B., *Pharmaceutical Chemistry Journal*, **2007**, 47(7), 362-366

8. **V. Belakhov**, Yu.D. Shenin, B.I. Ionin, Synthesis of Hydrophosphoryl Derivatives of the Antifungal Antibiotic Pimaricin by the Kabachnik-Fields Reaction, *Russian Journal of General Chemistry*, **2008**, 78(2), 305-312

9. V. Pokrovskaya, **V. Belakhov**, M. Hainrichson, S. Yaron, T. Baasov, Design, Synthesis and Evaluation of Novel Fluoroquinolone-Aminoglycoside Hybrid Antibiotics, *Journal of Medicinal Chemistry*, **2009**, 52(8), 2243-2254

10. I. Nudelman, A. Rebibo-Sabah, M. Cherniavsky, **V. Belakhov**, M. Hainrichson, F. Chen, J. Schacht, D.S. Pilch, T. Ben-Yosef, T. Baasov, Development of Novel Aminoglycoside (NB54) with Reduced Toxicity and Enhanced Suppression of Disease-Causing Premature Stop Mutations, *Journal of Medicinal Chemistry*, **2009**, 52(9), 2836-2845